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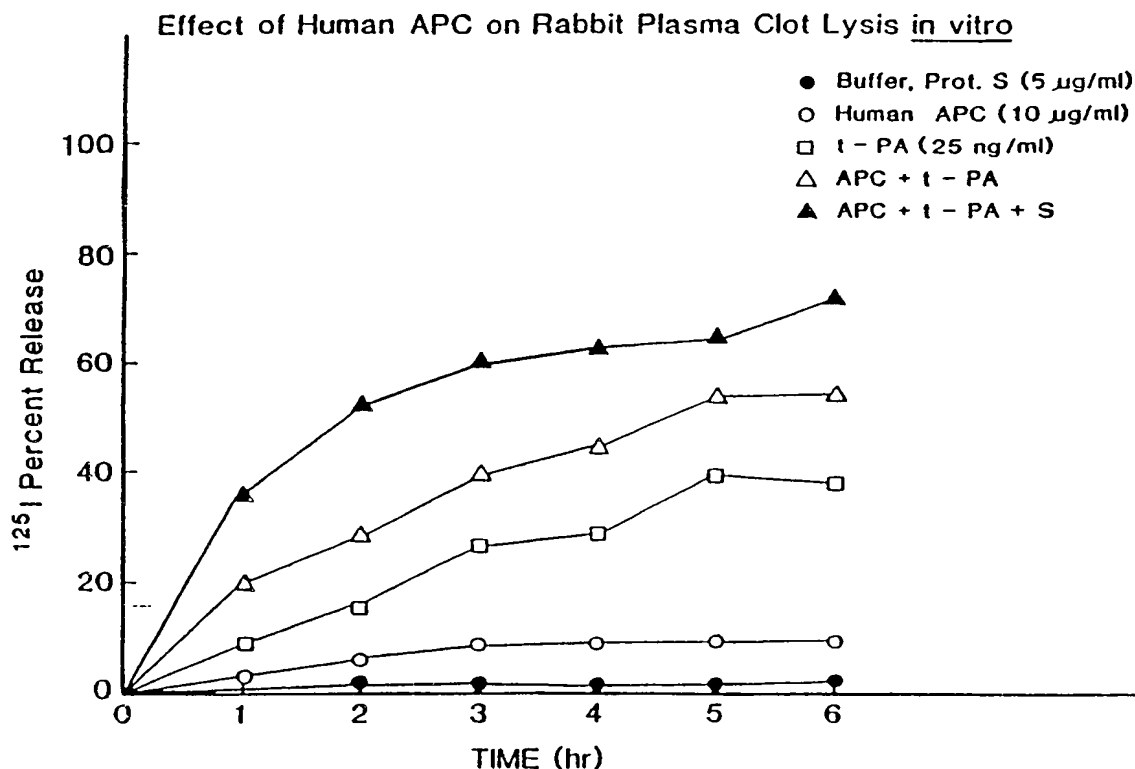
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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: <b>PCT/US88/03192</b> (22) International Filing Date: <b>15 September 1988 (15.09.88)</b>  (31) Priority Application Number: <b>104,240</b> (32) Priority Date: <b>5 October 1987 (05.10.87)</b> (33) Priority Country: <b>US</b>  (71) Applicant: <b>THE AMERICAN NATIONAL RED CROSS [US/US]; 400 Seventeenth Street, N.W., Washington, DC 20000 (US).</b>  (72) Inventors: <b>BHATTACHAYRA, Prabir ; 516 Carousel Court, Gaithersburg, MD 20877 (US). DROHAN, William, N. ; 8417 Oakford Drive, Springfield, VA 22151 (US). HOYER, Leon, W. ; 6 Bolling Lane, Bethesda, MD 20817 (US).</b>		(74) Agents: <b>STERN, Marvin, R. et al.; Holman &amp; Stern, 2401 Fifteenth Street, N.W., Washington, DC 20009 (US).</b>  (81) Designated States: <b>AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</b>  Published <i>With international search report.</i>

(54) Title: THERAPEUTIC THROMBOLYTIC COMPOSITION



(57) Abstract

A new therapeutic combination of t-PA and APC provides protection against blood clot reformation.

***FOR THE PURPOSES OF INFORMATION ONLY***

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1 TITLE: THERAPEUTIC THROMBOLYTIC COMPOSITION

2 BACKGROUND OF THE INVENTION

3 Technical Field:

4 The present invention is related generally to the  
5 field of thrombolytic agents. More specifically the  
6 present invention is related to the combination of two  
7 fibrinolytic agents, Tissue Plasminogen Activator (t-PA)  
8 and Activated Protein C (APC), to produce a unique  
9 therapeutic thrombolytic composition.

10 State of the Art:

11 Tissue Plasminogen Activator is a trace plasma  
12 protease with a molecular weight of about 72,000 which  
13 plays a central role in a number of physiological  
14 processes. Of these, its fibrinolytic activity has  
15 received the most attention. Its fibrinolytic activity  
16 is believed to involve its affinity for fibrin and its  
17 ability to form a fibrin/t-PA complex on the surface of  
18 recently-formed fibrin clots. Once formed, the  
19 fibrin/t-PA complex activates plasminogen to form  
20 plasmin, an active thrombolytic agent. To date, at least  
21 two fast-acting inhibitors of t-PA, Plasminogen Activator  
22 Inhibitor (PAI) I and II, have been identified in plasma

1 and have been shown to inactivate t-PA through complex  
2 formation (Sprengers and Kluft, Blood 69:38, 1987). In  
3 the presence of PAI I and II, t-PA exhibits a half-life  
4 of only two to three minutes in the circulatory system.

5 Like t-PA, Protein C is also a trace plasma  
6 protease zymogen. Unlike t-PA, when activated to an  
7 active protease, APC acts as a potent and specific  
8 anticoagulant whose activity has been described by many  
9 authors (Stenflo, J. Biol. Chem. 251:355, 1976; Kisiel et  
10 al, Biochem. 16:5824, 1977; Marlar et al, Blood 59:1067,  
11 1982). The mechanism of its anticoagulant effect  
12 involves the proteolytic cleavage of Factors Va and VIIIa  
13 to form inactive enzymes. Unlike t-PA, APC's activity  
14 appears to be species specific. It has been reported  
15 that bovine APC, despite considerable amino acid sequence  
16 homology with human APC, has negligible anticoagulant  
17 activity in human plasma (Kisiel, J. Clin. Invest.  
18 64:761, 1979; Walker Throm. Res. 22:321, 1981). Although  
19 the exact mechanism of APC's species specificity is not  
20 known, a possible explanation has been proposed by Walker  
21 supra. that the negligible anticoagulant activity is due  
22 to a lack of interaction between the bovine APC and human  
23 Protein S.

24 In addition to its anticoagulant activity,  
25 recently it has been demonstrated that bovine and human  
26 APC can act as a profibrinolytic agent. Several  
27 investigators have demonstrated elevated t-PA activity  
28 following infusion of APC into animals (Comp & Esmon, J.  
29 Clin. Invest. 68:1221, 1981; Burdick and Schaub Throm.  
30 Haem. 45:413, 1987). APC's profibrinolytic activity,  
31 like its anticoagulant activity, is highly species  
32 specific (Walker supra.), but the species specificity can

1        apparently be overridden with concomitant use of the  
2        autologous form of Protein S, APC's cofactor (Walker,  
3        supra). Although the mechanism by which APC exerts its  
4        profibrinolytic effect is unclear, it has been shown that  
5        APC can form a complex with PAI, thereby neutralizing PAI  
6        activity (Sakata et al, Blood 86:1218, 1986). These same  
7        authors have also demonstrated that t-PA itself is able  
8        to dissolve clots in a clot lysis assay. It should be  
9        noted, however, that in these experiments the addition of  
10       APC did not change either (1) the kinetics or (2) the  
11       quantity of t-PA required for maximum clot lysis within  
12       the experimental time period (20 hours). It should be  
13       noted that the addition of PAI did significantly inhibit  
14       clot dissolution by t-PA in the absence of APC.  
15       Importantly, PAI had no effect on clot dissolution when  
16       excess APC was present along with t-PA. In the same  
17       study, it was reported that human APC was less effective  
18       than its bovine counterpart in dissolving clots in human  
19       plasma.

20                In yet another study, Taylor et al, (Throm. Res.  
21        37:639, 1985) showed a dose-dependent profibrinolytic  
22        effect of human APC in an in vitro clot lysis assay.  
23        These investigators demonstrated approximately 65 percent  
24        clot lysis at 25-30 hours with human APC (20 µg/mL) in  
25        human whole blood. However, under the same conditions  
26        the authors were unable to demonstrate clot lysis in  
27        either citrated human plasma or platelet poor plasma.

28                Currently t-PA is undergoing clinical evaluation  
29        as a thrombolytic agent at doses approaching 100 to 150  
30        mg per patient. Bleeding, observed in approximately 20  
31        percent of those treated with t-PA, is the most common  
32        side effect and appears to be dose related. Consequently,

1 reducing the dose of t-PA administered to patients with  
2 thrombus formation is a crucial goal of this approach to  
3 thrombolytic therapy.

4 SUMMARY OF THE INVENTION

5 It is, therefore, an object of the present  
6 invention to combine two previously-described proteins,  
7 t-PA and APC, to produce a unique therapeutic  
8 thrombolytic composition with fibrinolytic and  
9 anticoagulant activity.

10 It is another object of the present invention to  
11 provide a method for inducing blood clot dissolution  
12 comprising contacting the blood clot with the composition  
13 of the present invention for sufficient time to produce  
14 thrombolytic effect and protection against blood clot  
15 reformation.

16 It is a further object of the present invention to  
17 reduce the dose of t-PA (recombinant or mammalian)  
18 required for effective thrombolysis and minimize the  
19 incidence of side effects thereof.

20 Other objects and advantages of the present  
21 invention will become evident from the Detailed  
22 Description of the Invention.

23 BRIEF DESCRIPTION OF THE DRAWINGS

24 These and other objects, features and many of the  
25 attendant advantages of the invention will be better  
26 understood upon a reading of the following detailed



description when considered in connection with the accompanying drawings wherein:

Fig. 1 shows the results of various treatments on blood clot dissolution;

Fig. 2 shows the results of various treatments on blood clots lysis using a radiolabeled marker; and

Fig. 3 shows the effect of human APC in combination with t-PA on rabbit plasma clot lysis with or without Protein S.

#### DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the invention are achieved by a thrombolytic or anticoagulant composition, comprising an admixture of tissue Plasminogen Activator (t-PA) and activated Protein C (APC), each in an amount substantially less than that required for t-PA or APC alone to produce thrombolytic or anticoagulant effect.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

1           In general the following methods were used. Human  
2   pooled plasma was clotted at room temperature  
3   (about 22°-25°C) by addition of of human thrombin and the  
4   resultant clot was suspended in 25mM Tris buffer  
5   containing 0.1 M NaCl and 5mM CaCl<sub>2</sub> pH 7.5. The  
6   concentrations of human APC (4.5 µg/mL) and t-PA (50  
7   ng/mL) used for the clot lysis assay are only  
8   illustrative. After adding the test agents, all samples  
9   were incubated at 37°C. In all examples cited below,  
10   clot lysis was maximal when the combination of t-PA and  
11   APC was used. Since different diseases may respond  
12   differentially to different ratios of the two agents  
13   (t-PA and APC), it is clear that a variety of therapeutic  
14   regimens comprising different ratios of these two  
15   pharmacologic agents can be formulated to treat specific  
16   condition(s). Because t-PA is not known to be species  
17   specific, either single or 2-chain t-PA from any source  
18   could be employed. Since APC's species specificity is  
19   most probably due to its interaction with the autologous  
20   protein, Protein S, it is apparent that in place of human  
21   APC, one could substitute a combination of APC and  
22   Protein S as long as they are obtained from the same  
23   species or obtained by recombinant technology.

#### 24           E X A M P L E I

25           In order to demonstrate that the combination of  
26   APC and t-PA exhibits unexpected synergistic or enhanced  
27   fibrinolytic activity, a simple in vitro experiment was  
28   designed to establish that the combination is more  
29   effective in dissolving blood clots than the individual  
30   components at the same concentration or dosage level.  
31   In this test, clots were formed in each of the four test  
32   tubes by adding 100 µL of 25 mM CaCl<sub>2</sub> and 10 µL of

thrombin (1  $\mu$ M) to 0.5 mL pooled human plasma. The time required for clot dissolution was measured following the addition of 500  $\mu$ L of one of the following four reagents: (1) Tris/NaCl buffer 5mM  $\text{CaCl}_2$ , (2) 20  $\mu$ L APC (5  $\mu$ g) and 25  $\mu$ L t-PA (0.05  $\mu$ g), (3) 20  $\mu$ L APC (5  $\mu$ g) and (4) 25  $\mu$ L t-PA (0.05  $\mu$ g). Table 1 shows the results obtained which were as follows.

Clot dissolution was minimal in the test tubes treated with Tris/NaCl 5mM  $\text{CaCl}_2$  buffer or APC as a single agent (test tubes 1 and 3); clot dissolution was complete within 20 hours with single agent t-PA (test tube 4); the combination of t-PA and APC results in 80 percent dissolution at three hours with dissolution complete at four hours (test tube 2). Figure 1 shows the results obtained at three hours.

T A B L E 1

Test Tube #	Agents	Clot Dissolution (%) at 3 Hrs.
1	Buffer	<10
2	5 $\mu$ g APC + 0.05 $\mu$ g t-PA	80 - 90
3	Human APC (5 $\mu$ g)	10 - 20
4	t-PA (0.05 $\mu$ g)	40 - 50

E X A M P L E   I I

A second experiment was designed to more accurately quantitate the results. The test was conducted using clots formed essentially as described in Example I but which were in addition radiolabeled by incorporating about 770,000 cpm of  $^{125}\text{I}$ -fibrinogen into each clot. As shown in Panels A, B and C of Figure 2, these clots were then treated in one of the following ways: (1) with 4.5  $\mu\text{g}$  of single agent APC (open circle); (2) one of three concentrations of single agent t-PA represented by the square (Panel A - 0.0125, Panel B - 0.025 and Panel C - 0.05  $\mu\text{g}$ ) and (3) combinations of APC and t-PA represented by the triangles at their corresponding single agent concentrations. Clot dissolution was quantitated with a gamma counter by counting 25  $\mu\text{L}$  aliquots from each test tube at hourly intervals beginning at time 0 and continuing for six hours. As shown in Figure 2, these data indicate that when APC at 4.5  $\mu\text{g}$  is combined with t-PA, at 50 ng/mL, the combination yields complete clot dissolution at one-half of the t-PA concentrations (Panel B) required by single agent t-PA (Panel C) for complete lysis in the same period of time. Table 2 summarizes these data.

T A B L E 2

Agents	$^{125}\text{I}$ Released (%) at 6 Hrs.
Human APC	06
t-PA (12.5 ng/mL)	31
t-PA (25 ng/mL)	38
t-PA (50 ng/mL)	80
APC + t-PA (12.5 ng/mL)	68
APC + t-PA (25 ng/mL)	85
APC + t-PA (50 ng/mL)	88

E X A M P L E III

To further elucidate the conditions of APC's species specificity, a third in vitro test was conducted using clots formed in rabbit plasma. To form the clots, 0.1 mL of 25 mM  $\text{CaCl}_2$  and 10  $\mu\text{L}$  of  $^{125}\text{I}$ -fibrinogen (about 500,000 cpm) were added to 0.5 mL of citrated rabbit plasma. The clots were initiated by the addition of 10  $\mu\text{L}$  of 1  $\mu\text{M}$  human thrombin. The resultant clot was suspended by addition of 0.5 mL of 25mM Tris buffer containing 0.1M NaCl and 5mM  $\text{CaCl}_2$ , pH 7.5. As shown in Figure 3, clot lysis was measured following the addition of (1) buffer and human Protein S (5  $\mu\text{g}/\text{mL}$ ); (2) single agent t-PA (25 ng/mL); (3) t-PA (25 ng/mL) in combination with human APC (10  $\mu\text{g}/\text{mL}$ ) and (4) t-PA (25ng/mL), human APC (10  $\mu\text{g}/\text{mL}$ ) and human Protein S (5  $\mu\text{g}/\text{mL}$ ). At hourly intervals, 25 mL aliquots of supernatant were withdrawn and counted in a gamma counter. Results, as shown in Figure-3, indicate that, t-PA in combination with human APC and its cofactor human S, produced greater than 50 to 70 percent clot lysis at 2 and 6 hours, respectively. However, the same combination in the absence of human S

produced only 20 to 50 percent clot lysis. These results clearly indicate that species specificity of APC can be overcome by using autologous Protein S (See Table 3).

T A B L E 3

Agents	<sup>125</sup> I Released (%) at 6 Hrs.
Buffer, Protein S (5 µg/mL)	01
Human APC (10 µg/mL)	10
T-PA (25 ng/mL)	37
10 µg APC + 25 ng/mL t-PA	52
10 µg APC + 25 ng/mL t-PA + Protein S (5 µg/mL)	70

Since, the side effect of hemorrhage encountered with t-PA at the currently recommended clinically effective dosage level of about 100-150 mg per patient, is a serious problem in its use as a thrombolytic agent, the present invention, for the first time, makes it possible to alleviate this dangerous side-effect by reducing the dosage level of t-PA while still achieving effective thrombolysis when such reduced dosage level of t-PA is combined with APC, optionally with autologous Protein S. In addition, reducing the t-PA level needed for effective treatment also reduces the cost of this type of therapy.

For the practice of the present invention, APC or t-PA can be obtained synthetically, by recombinant genetic technology, from mammalian source or by any other means or method and either the whole molecule or, a biologically active part thereof can be employed so long as substantially similar results are obtained.

1           It should be noted that an important additional  
2 advantage of the present invention is that the  
3 combination of APC and Protein S provides the protection  
4 against arterial reocclusion due to rethrombosis, since  
5 the combination of APC and Protein S is known to have  
6 potent anticoagulant and profibrinolytic activity.  
7 Restenosis is a major problem with patients receiving  
8 t-PA therapy for clot dissolution. Furthermore, APC +  
9 protein S should be useful in reducing the rate of  
10 restenosis in any patient, in which this has been shown  
11 to be a problem.

12           The synergistic effect obtained with the  
13 therapeutic combination as demonstrated herein opens a  
14 new vista for the treatment of those conditions which  
15 result from thrombus formation. Of course, the  
16 composition of the present invention can be administered  
17 in any pharmaceutically acceptable vehicle, if necessary,  
18 such as non-toxic sterile buffer, physiological saline  
19 and the like and it can be administered through any  
20 suitable route such as orally, intramuscularly,  
21 intravenously and the like.

22           It is understood that the examples and embodiments  
23 described herein are for illustrative purposes only and  
24 that various modifications or changes in light thereof  
25 will be suggested to persons skilled in the art and are  
26 to be included within the spirit and purview of this  
27 application and scope of the appended claims.

1     WHAT IS CLAIMED IS

2             1. A thrombolytic or anticoagulant composition,  
3 comprising an admixture of tissue Plasminogen Activator  
4 (t-PA) and activated Protein C (APC), in which t-PA is in  
5 an amount substantially less than that required for t-PA  
6 alone to produce thrombolytic effect, said composition  
7 being effective either as a fibrinolytic or  
8 anticoagulant.

9             2. The composition of claim 1 further comprising  
10 autologous Protein S.

11            3. The composition of claim 1 wherein the APC is  
12 in an amount, at a constant t-PA concentration, which  
13 reduces the time required for thrombolysis.

14            4. A method for blood clot dissolution comprising  
15 contacting blood clot with an effective amount of the  
16 composition of claim 1 for sufficient time to produce  
17 thrombolytic or anticoagulant effect.



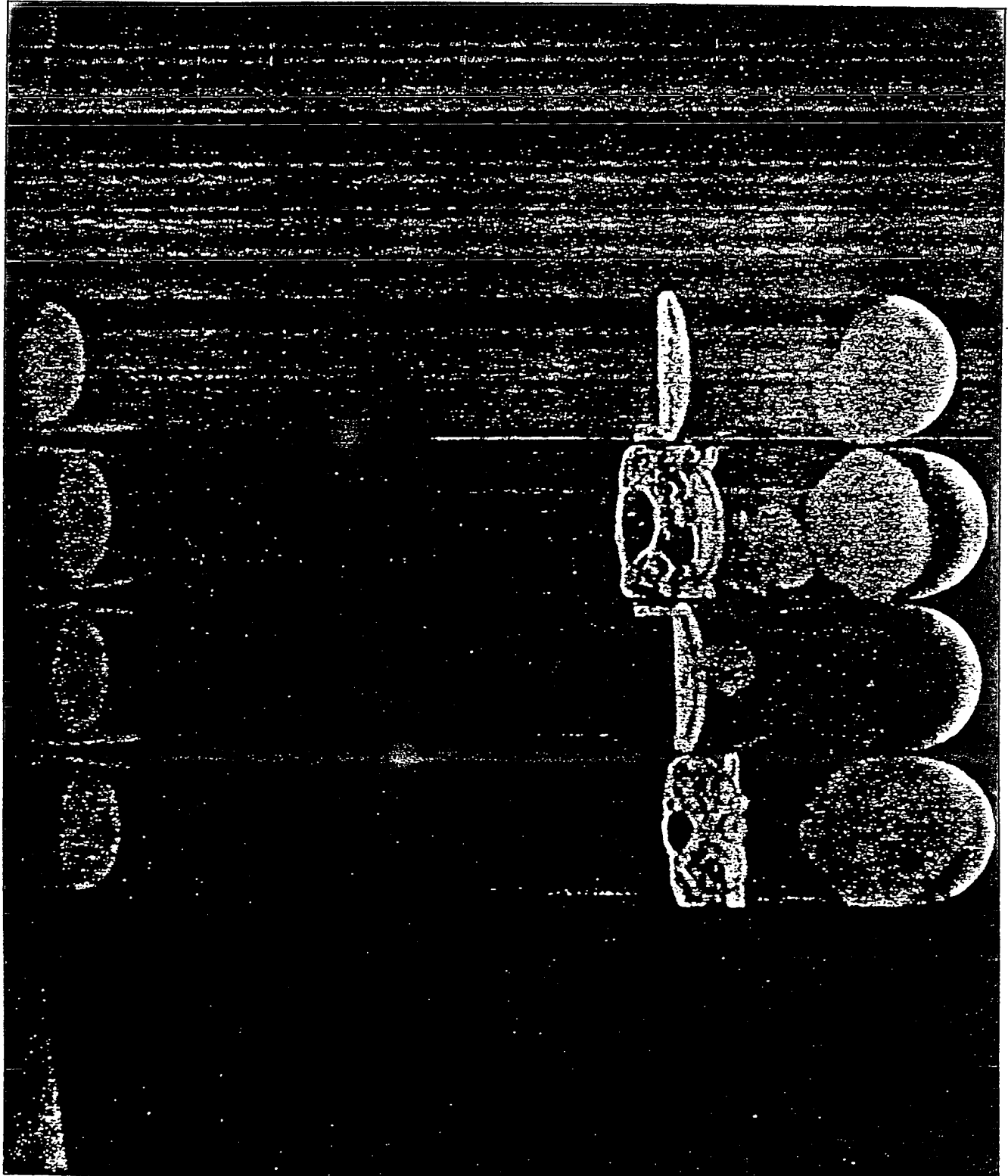


FIG. 1

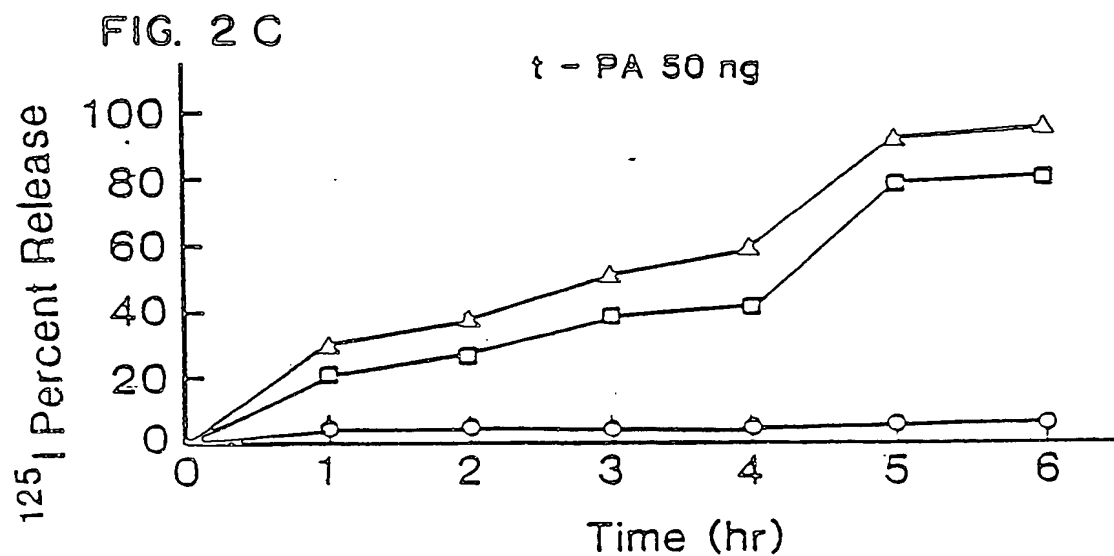
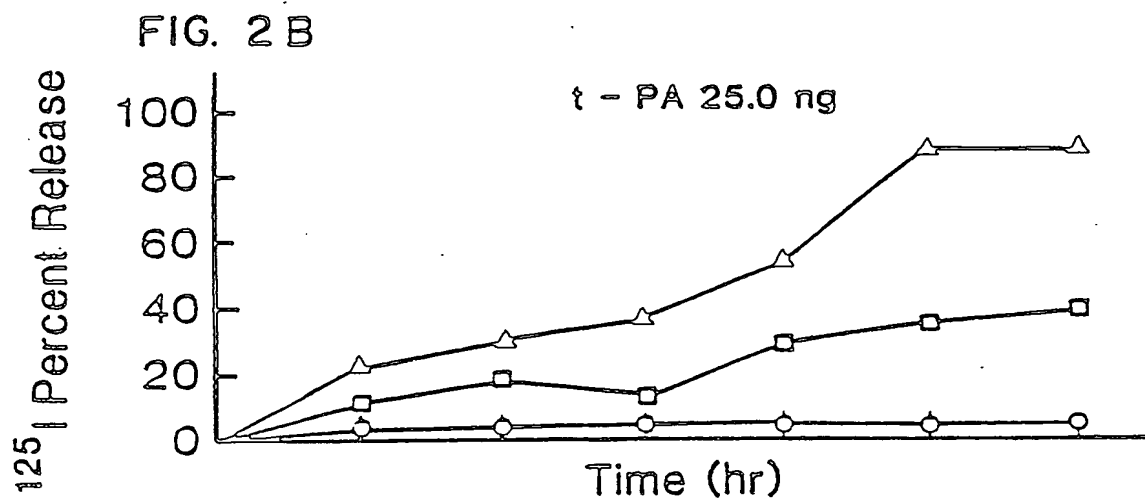
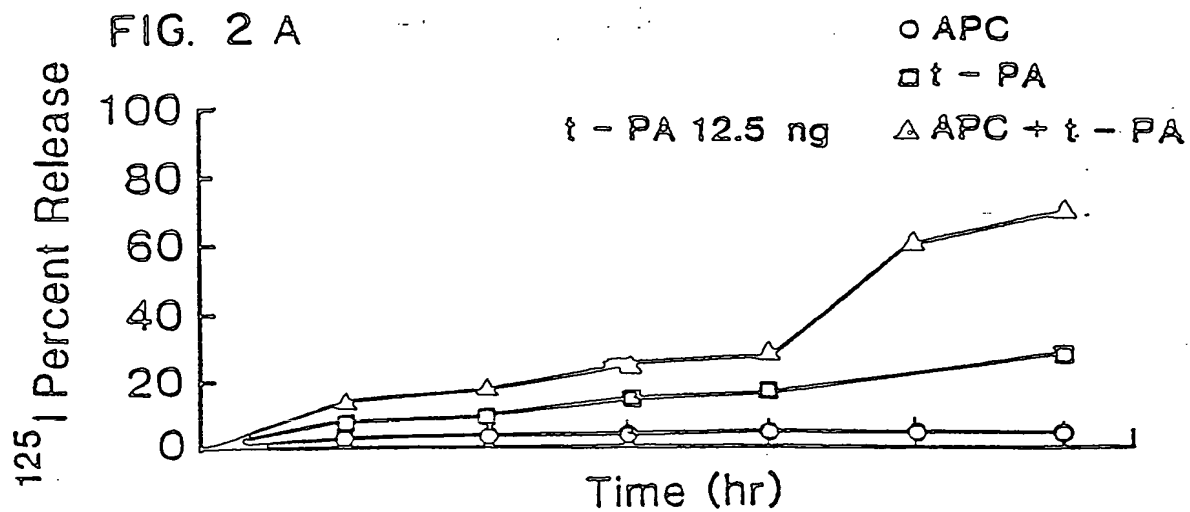
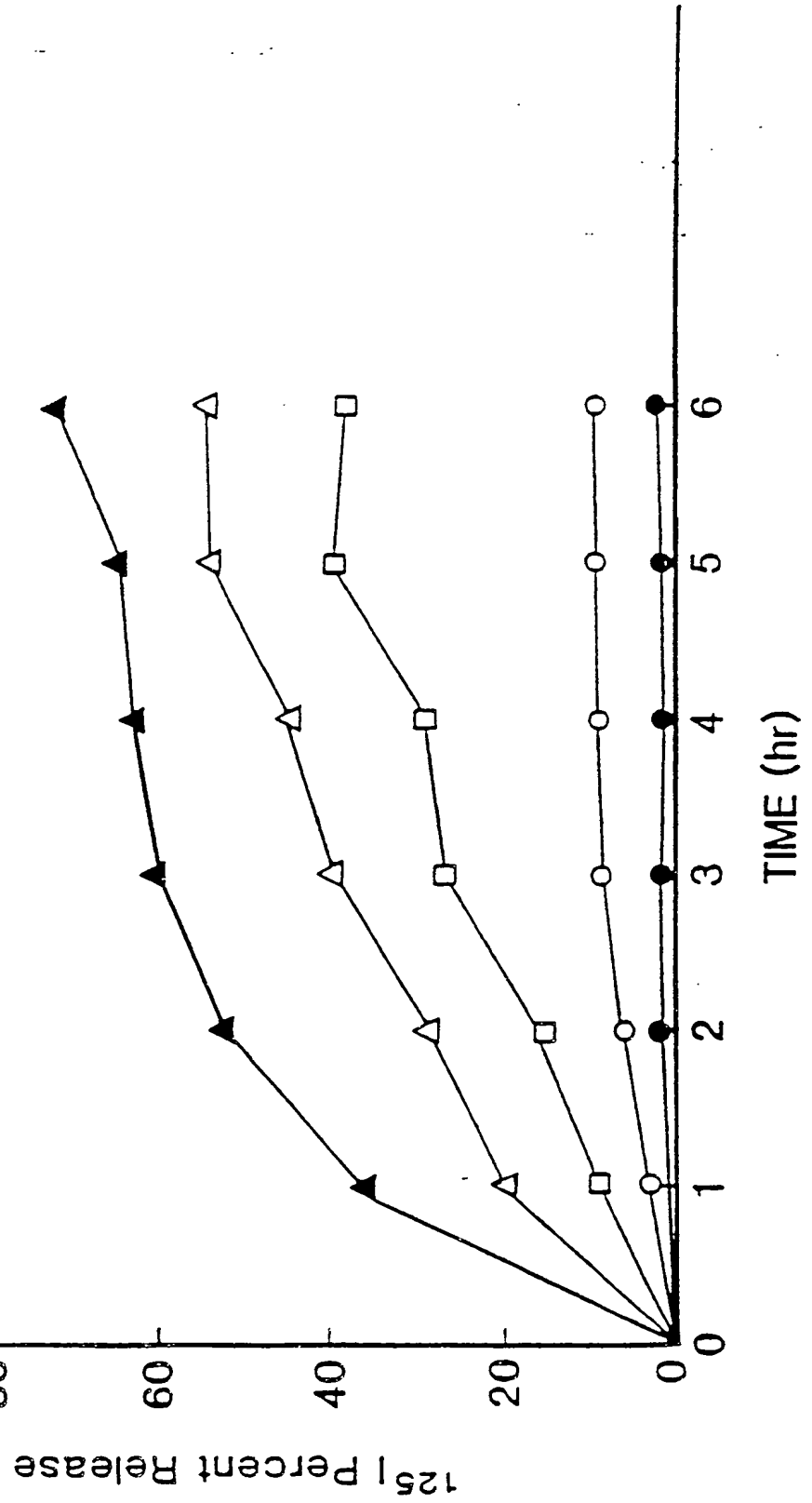


FIG. 3 Effect of Human APC on Rabbit Plasma Clot Lysis in vitro

- Buffer, Prot. S (5  $\mu$ g/ml)
- Human APC (10  $\mu$ g/ml)
- t - PA (25 ng/ml)
- △ APC + t - PA
- ▲ APC + t - PA + S



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	COMP ET AL, "Generation of Fibrinolytic Activity by Infusion of Activated Protein C into Dogs", Journal of Clinical Investigation, issued November 1981, volume 68, 1221-1228, see the entire document.	1, 3 and 4
Y	US, A, 4,552,760, (MURAKAMI ET AL), 12 November 1985, see columns 5 and 6.	1, 3 and 4

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03192

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.: 424/94.2, 94.63

IPC<sup>4</sup>: A61k 37/48; C12N 9/48

## II. FIELDS SEARCHED

Minimum Documentation Searched <sup>7</sup>

Classification System	Classification Symbols
U.S.	424/94.2, 94.64, 101; 435/212; 530/380; 514/2,21

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>

CASONLINE, BIOSIS

## III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	DE FOUW ET AL, "The Cofactor Role of Protein S in the Acceleration of Whole Blood Clot Lysis by Activated Protein C In Vitro", Blood, issued April 1986, volume 67, number 4, 1189-1192, see the entire document.	1-4
Y	TAYLOR ET AL, "A New Function For Activated Protein C: Activated Protein C Prevents Inhibition of Plasminogen Activators by Releaseate from Mononuclear Leukocytes-Platelet Suspensions Stimulated by Phorbol Diester", Thrombosis Research, Issued 1985, volume 37, number 1, 155-164, see pages 155 and 162-163.	1, 3 and 4

\* Special categories of cited documents: <sup>10</sup>

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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## IV. CERTIFICATION

Date of the Actual Completion of the International Search

11 November 1988

Date of Mailing of this International Search Report

11 JAN 1989

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*J. M. Stone*  
J. M. Stone

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